

Influenza Virus Receptor Specificity and Cell Tropism in Mouse and Human Airway Epithelial Cells

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Recent human infections caused by the highly pathogenic avian influenza virus H5N1 strains emphasize an urgent need for assessment of factors that allow viral transmission, replication, and intra-airway spread. Important determinants for virus infection are epithelial cell receptors identified as glycans terminated by an α 2,3-linked sialic acid (SA) that preferentially bind avian strains and glycans terminated by an α 2,6-linked SA that bind human strains. The mouse is often used as a model for study of influenza viruses, including recent avian strains; however, the selectivity for infection of specific respiratory cell populations is not well described, and any relationship between receptors in the mouse and human lungs is incompletely understood. Here, using in vitro human and mouse airway epithelial cell models and in vivo mouse infection, we found that the α 2,3-linked SA receptor was expressed in ciliated airway and type II alveolar epithelial cells and was targeted for cell-specific infection in both species. The α 2,6-linked SA receptor was not expressed in the mouse, a factor that may contribute to the inability of some human strains to efficiently infect the mouse lung. In human airway epithelial cells, α 2,6-linked SA was expressed and functional in both ciliated and goblet cells, providing expanded cellular tropism. Differences in receptor and cell-specific expression in these species suggest that differentiated human airway epithelial cell cultures may be superior for evaluation of some human strains, while the mouse can provide a model for studying avian strains that preferentially bind only the α 2,3-linked SA receptor.

An important aspect of influenza virus infection of airway epithelial cells is the interaction between the virus hemagglutinin (HA) protein and the corresponding receptor on the host cell. Although the precise nature of the viral receptor is incompletely defined, influenza viruses target glycosylated oligosaccharides that terminate in a sialic acid (SA) residue (9, 35, 41). These residues are bound to glycans through α 2,3, α 2,6, or α 2,8 linkage by sialyltransferases that are expressed in a cell- and species-specific manner (1, 11). Influenza viruses primarily target airway epithelial cells via α 2,3- and α 2,6-type receptors, but the distribution of these receptors in many species is uncertain and may be a significant factor influencing infection. For example, influenza A viruses isolated from avian species preferentially bind to SA receptors that are linked to galactose by Neu5Ac α 2,3Gal residues, while human strains preferentially bind the Neu5Ac α 2,6Gal-terminated sugar chains (9, 35, 41).

The preference of receptor binding can be altered by changes in specific HA amino acids that can influence host specificity and tropism (3, 34). Indeed, mutations of avian influenza virus HA genes have been shown to alter binding preference from the α 2,3- to the α 2,6-linked SA and are thought to have contributed to the 1918 Spanish, 1957 Asian, and 1968 Hong Kong pandemics (3, 34, 53). Thus, analysis of HA genomic sequences and SA-specific binding has been used

to predict the risk of human infection (15, 16, 41). However, it has been recognized recently that avian strains may infect humans directly, without undergoing HA gene mutation or strain reassortment, as occurred in the 1997 and 2003-to-2005 avian influenza outbreaks in China and Vietnam (4, 5). This suggests that not only mutations in HA but also mutations in other viral genes, such as NS1 and PB2, may be important determinants of infectivity (28, 43).

To address these issues, virus-epithelial cell interactions and newly emerging strains of influenza virus are often assayed in epithelial cell lines and mouse models. Though many cell lines are capable of supporting influenza virus infection (17, 21, 39, 47), compared to primary respiratory epithelial cells, continually passaged cells are transformed and therefore have different genetic programs regulating important features such as localization of receptors and innate host responses (50). Furthermore, the epithelial cells of the airway are not a homogeneous cell population but are composed of secretory (Clara), goblet (mucous), ciliated, basal, and other cell types that differ in frequency and distribution between species (20). Thus, respiratory epithelial cells cultured under physiologic conditions that allow for full differentiation into types of cells representative of the native airway may provide a better infection model. In addition to in vitro approaches, it is also critical to extend findings to an in vivo model. The mouse has historically been used for studying influenza viruses (19, 57), and genetically engineered mice have been particularly valuable for understanding adaptive immune responses (49, 58). However, mice differ in their susceptibilities to human virus strains (25, 48). Many factors may be responsible for this observation,

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including virus-specific receptor expression in the mouse lung. Receptor expression in the mouse compared to the human airway is incompletely defined, and the susceptibility of specific respiratory cell types to influenza virus is not well described.

The present study was therefore designed to better define the interaction of influenza virus with specific populations of mouse compared to human airway epithelial cells *in vitro* and *in vivo*. We found that the α 2,3-linked SA receptor was expressed selectively on the apical membranes of mouse and human ciliated airway epithelia and type II alveolar epithelial cells. This cell-specific localization explained the capacity of a virus that selectively binds the α 2,3-linked SA receptor to preferentially infect these cells *in vitro* and *in vivo*. In contrast, the α 2,6-linked receptor was not found in the mouse lung, and in the human lung it was restricted to ciliated and goblet cells. Understanding patterns of influenza virus receptor expression in specific populations of mouse and human airway epithelial cells can enhance the understanding of established and emerging influenza virus strains causing human infections.

MATERIALS AND METHODS

Cell culture. MDCK cells (Madin-Darby canine kidney cells; American Type Culture Collection [ATCC], Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics. MDCK cells were polarized by culture on semipermeable Transwell membranes (Corning-Costar, Corning, NY). When cells were confluent, medium was removed from the apical compartment and cells were maintained in culture for at least 7 days. Mouse tracheal epithelial cells (mTEC) were obtained from C57BL/6J mice. Human tracheal epithelial cells (hTEC) were obtained from tracheas of lung transplantation donors ($n = 7$) by using a protocol approved by the institutional review board at the Washington University School of Medicine. Tracheas were treated with pronase and epithelial cells purified by differential adherence on plastic (60). hTEC and mTEC cultures were established on Transwell membranes using air-liquid interface (ALI) conditions as previously described (60). Cells were grown in "basic medium," composed of DMEM-Ham's F-12 medium with 30 mM HEPES, 4 mM L-glutamine, 3.5 mM NaHCO₃, 100 U/ml penicillin, and 100 μ g/ml streptomycin, supplemented with 10 μ g/ml insulin, 10 μ g/ml transferrin, 0.1 μ g/ml cholera toxin, 25 ng/ml epithelial growth factor (Becton Dickinson, Bedford, MA), 30 μ g/ml bovine pituitary extract, 0.01 μ M retinoic acid, and 5% fetal bovine serum. Cells were continuously maintained in the antifungal agent amphotericin B (mTEC, 0.25 μ g/ml; hTEC, 0.50 μ g/ml). Medium was provided in the upper and lower chambers until the transmembrane resistance was $>1,000 \Omega \cdot \text{cm}^2$, corresponding to tight-junction formation (60). Then, to induce differentiation, the medium in the apical chamber was removed to create an ALI and the lower compartment medium was changed to basic medium supplemented with 2% Nuserum (BD BioSciences, San Diego, CA) and retinoic acid. When mature (after at least 7 days of ALI), the top layer of mTEC cultures was composed of 30 to 50% ciliated cells.

Virus infection of epithelial cells. Virus strains included influenza virus A/WSN/33 (H1N1; ATCC), A/California/7/2004 (H3N2; low passage; kindly provided by N. Cox, Centers for Disease Control and Prevention, Atlanta, GA), Sendai virus (SeV) strain 52 (Fushimi strain; ATCC VR-105), N31S-M2 WSN (rWSN) recombinant mouse-adapted influenza A virus (A/WSN/33 background) containing a serine-for-asparagine substitution at amino acid 31 of the M2 protein (46), recombinant A/Udorn/72 (rUdorn) (46), human parental A/HK/1/68 (H3N2), and the mouse-adapted derivative A/HK/1/68-MA20C (7). Prior to infection, cells were washed and placed in serum-free medium composed of basic medium supplemented with 5 μ g/ml insulin, 5 μ g/ml transferrin, 5 ng/ml epidermal growth factor, 30 μ g/ml bovine pituitary extract, 1 mg/ml bovine serum albumin (BSA), and 0.01 μ M retinoic acid. The virus concentration (and multiplicity of infection [MOI]) applied to the apical surface of cells (2×10^5 cells/insert) was as follows: for A/WSN/33 and rWSN, 1×10^6 PFU (MOI, 5); for A/California/7/2004, 2×10^6 PFU (MOI, 10); for Sendai virus, 1×10^5 PFU (MOI, 0.5); for rUdorn, 2×10^4 PFU (MOI, 0.1); and for A/HK/1/68 and A/HK/1/68-MA20C, 1×10^4 PFU (MOI, 0.05). Virus was incubated with cells for 1 to 24 h as indicated. Acetylated trypsin at 1 μ g/ml (Sigma, St. Louis, MO) was added to MDCK cells. Preliminary studies indicated that similar levels of virus production and hemagglutinin cleavage occurred in mTEC cultures in the pres-

ence or absence of trypsin; thus, trypsin was not used in primary culture of mouse or human epithelial cells. Following incubation of virus, cells were washed three times with warm phosphate-buffered saline (PBS; pH 7.4). Collection of apical media 1 h after washes showed no infectious virus by plaque assay.

Plaque and TCID₅₀ assays. The plaque assay was performed as previously described (46). Briefly, samples were 10-fold serially diluted in DMEM supplemented with 0.5% BSA, 2 μ g/ml acetylated trypsin, and penicillin-streptomycin, applied in duplicate to confluent MDCK cells, and incubated for 1 h at 25°C. The inoculum was removed, and the cells were first washed with PBS and then overlaid with DMEM containing 2% agarose and 4 μ g/ml acetylated trypsin. At day 3 after infection, the cells were stained with a solution containing 0.1% naphthalene black (Bio-Rad, Hercules, CA), 6% glacial acetic acid, and 1.66 mM anhydrous sodium. The 50% tissue culture infective dose (TCID₅₀) assay was performed in MDCK cells as previously described (37).

Immunohistochemistry. Cells on supported membranes were fixed with 4% paraformaldehyde in PBS for 10 min at 25°C and processed for immunodetection as described elsewhere (59, 60). For detection of γ -tubulin, cells were treated with PHEM buffer [45 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES), 45 mM HEPES, 10 mM EGTA, 5 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, pH 6.8] for 1 min prior to fixing. Nonspecific antibody binding was blocked using 3% BSA in PBS or 3% BSA in PBST (PBS with 0.2% Triton X-100) for 1 h at 25°C. Samples were then incubated for 1 h at 25°C or 18 h at 4°C with an isotype-matched control antibody or primary antibody. Primary antibodies and dilutions used were as follows: mouse anti-M2 (14C2 hybridoma), 1:500 (37); goat anti-hemagglutinin, 1:200 (recognizes H1 subtype); mouse anti- β -tubulin-IV, 1:250 (BioGenex, San Ramon, CA); rabbit anti- γ -tubulin, 1:1,000 (Sigma); mouse monoclonal anti-Foxj1, 1:500 (clone 1A4; N-terminal mouse foxj1 immunogen); rabbit anti-active caspase-3, 1:500 (BD Pharmingen, San Jose, CA); mouse anti-pro-surfactant protein C, 1:2,000 (Chemicon, Temecula, CA); mouse anti-T1 α , 1:250 (clone 8.1.1; Iowa Hybridoma Bank, Iowa City, IA); goat anti-human Clara cell secretory protein (CCSP), 1:50 (Santa Cruz Biotechnology, Santa Cruz, CA); and mouse anti-Muc5AC, 1:300 (clone 45M1; NeoMarkers, Fremont, CA). Antibody binding was detected using a whole immunoglobulin G or F(ab') fragment secondary antibody conjugated with fluorescein isothiocyanate (FITC), indocarbocyanine (CY3) (Jackson ImmunoResearch Laboratories, West Grove, PA), Alexa Fluor 633, or Alexa Fluor 488 (Molecular Probes, Carlsbad, CA). No detectable staining was observed for isotype-matched or species-specific control antibodies. Annexin V labeled with FITC was diluted in annexin V staining buffer (both from Biosource, Camarillo, CA) using the manufacturer's recommended concentration, incubated with cells on membranes for 30 min at 25°C, and then washed twice with PBS. Membranes were mounted on slides with medium (Vectashield; Vector Laboratories, Burlingame, CA) containing 4',6'-diamidino-2-phenylindole (DAPI) to stain intracellular DNA. Microscopy was performed using a Zeiss (Thornwood, NY) LSM 510 META laser scanning confocal microscope and an Olympus (Melville, NY) BX51 microscope for reflected fluorescent imaging with a charge-coupled device camera interfaced with MagniFire software (Olympus). Images were composed using Photoshop and Illustrator software (Adobe Systems, San Jose, CA).

Lectin binding and competition. Lectin from *Maaackia amurensis* (MAA) (EY Laboratories, San Mateo, CA, and Vector Laboratories) was used to bind α 2,3-linked SA, and *Sambucus niger* agglutinin (SNA) (Vector Laboratories) was used to bind α 2,6-linked SA (36, 44, 56). Lectins were purchased in nonconjugated and biotinylated forms. To assay lectin binding, cells were fixed for immunofluorescent staining as described above but were treated with 3% BSA in PBS (blocking buffer) for 1 h to block nonspecific binding. MAA (10 μ g/ml) or SNA (1 μ g/ml), diluted in blocking buffer, was incubated with cells at 4°C for 18 h. Biotinylated lectin binding was detected using streptavidin-Texas red (Vector Laboratories) or streptavidin-Alexa Fluor 555 (Molecular Probes). To detect the specificity of lectin binding, cells were pretreated with 20-fold excess nonconjugated lectin for 2 h at 25°C prior to incubation with biotinylated lectin. To determine the ability of lectin to block influenza virus infection, cells were pretreated with 1 mg/ml of nonconjugated lectin for 1 h at 37°C prior to inoculation with virus.

Infection of mice. C57BL/6J mice were obtained from Jackson Laboratory (Bar Harbor, ME) and maintained under pathogen-free conditions. The Animal Studies Committee approved all mouse study protocols. Viral inoculation and monitoring were performed as described previously (50, 55). For the present experiments, mice were inoculated intranasally with 10^2 , 10^3 , and 10^4 PFU of influenza A virus strain A/WSN/33 (H1N1) diluted in 30 μ l of sterile PBS. Mortality rate and weight loss were measured daily as previously described (55). For analysis of virus infection, lungs were harvested as previously described (29). The right lung was inflation fixed for immunohistochemistry, and the left lung was homogenized for assay of virus production.

Lung tissue sections. Mouse lungs were inflation fixed with 10% buffered formalin at 20 cm, embedded in paraffin, and sectioned at 5 μ M. Human lung sections were obtained from uninvolved portions of surgical resection samples from patients undergoing removal of lung tissue for possible carcinoma, and regions containing carcinoma or infection were excluded. For peroxidase-based detection of lectin binding, endogenous peroxidase was inactivated by incubation in 4.5% H_2O_2 in PBS for 5 min. Tissues were incubated with lectins or primary antibodies using the method utilized for cells on membranes described above. Thereafter, samples were washed and incubated with biotinylated secondary antibodies bound with peroxidase and detected with 3,3'-diaminobenzidine (DAB) substrate solution (ABC Elite; Vector Laboratories), or fluorescent dyes as described above. Immunoperoxidase-stained samples were counterstained with hematoxylin.

Statistical analysis. Means of cell numbers were compared using Student's *t* test. Multiple groups were analyzed using a one-way analysis of variance (ANOVA). If significance was achieved by one-way analysis, post-ANOVA comparison of means was performed using Scheffe's *F* test. Mouse mortality was analyzed using Kaplan-Meier estimates of survival. The equality of survivorship distribution function for each group was determined by the Wilcoxon rank-sum test. The level of significance for all analyses was <0.05 .

RESULTS

Influenza virus production and survival of MDCK cells compared to mouse tracheal epithelial cells. MDCK cells are commonly used to study respiratory virus-epithelial cell interactions (21, 39); however, it is difficult to extrapolate findings with these cells to the effects viruses might have on primary airway cells. We focused initially on the airway epithelial cells, since these were considered to be the major target for infection of most human influenza virus strains. Accordingly, mTEC were cultured using ALI conditions to generate a high-fidelity model of mouse epithelium composed of ciliated and nonciliated cells (60). In initial studies, MDCK cells and mTEC cultures were incubated with influenza virus (rWSN). Within 2 days, the two cell types had similar peaks in virus production that subsequently decreased rapidly (Fig. 1A). In MDCK cells, the fall in virus production could be explained by a rapid decrease in cell number and death of the entire sample by postinoculation day 4, whereas in mTEC cultures, the decrease was less marked (Fig. 1B). To determine if mTEC death might be delayed, we followed cells for an additional 1-week period. During this time, cell junctions remained intact, as indicated by maintenance of high transmembrane resistance (data not shown) across the cell layer (60). Although not as marked as the loss of MDCK cells (Fig. 1B), the number of mTEC treated with virus significantly decreased after 4 days (Fig. 1C). During this period, the percentage of cells that expressed influenza virus protein M2 also significantly decreased (Fig. 1D). These findings led us to consider that the surviving mTEC could not support influenza virus infection over a prolonged period.

mTEC cultures are resistant to reinfection *in vitro*. To further study the relative resistance of mTEC cultures to infection, we attempted to reinfect mTEC at 10 days after the initial inoculation. We chose this time because there was no further loss of cells after day 7 and the level of M2 expression remained low. Compared to naive cells, repeat exposure to virus in the previously infected cells resulted in low levels of influenza virus M2 expression and virus production (Fig. 1E and F). These findings raised the possibility that either cells surviving the first infection or new susceptible cells generated within the culture system were now infected. Both of these

possibilities suggested that a specific cell type was targeted for viral infection.

Influenza virus infection is selective for ciliated mouse airway epithelial cells. Loss of airway epithelial cells is known to occur in mice infected with influenza virus; however, injury of a specific population of airway epithelial cells has not been defined (40, 57). In this regard, immunostaining for cell-specific proteins after infection with H1N1 virus (rWSN and A/WS/33; MOI, 5) revealed a progressive loss of ciliated cells, identified by the cilium-enriched protein β -tubulin-IV (Fig. 2A and B). The relationship between virus infection and the selective loss of ciliated cells was additionally established by several approaches. Selective infection of ciliated cells was identified by colocalization of influenza virus protein M2 with β -tubulin-IV (Fig. 2C). Virus-induced death of ciliated cells was demonstrated by colocalization of M2 and the apoptosis markers activated caspase-3 and annexin V with ciliated cell markers (Fig. 2D). Increasing the MOI did not change the cell type specificity (i.e., nonciliated cells were not infected), and use of a MOI above 50 did not increase the number of infected ciliated cells or the amount of virus produced (Fig. 2E). Furthermore, infection of mTEC cultures with an additional mouse-adapted strain (A/HK/1/68-MA20C), compared to its parental human strain (A/HK/1/68), resulted in a greater number of infected ciliated cells (19.0% versus 0.6%), but no difference in cell-type selectivity was observed. When a 10-fold-higher titer of the parental strain was used, the number of infected cells increased; however, again, there was no change in cell specificity of infection. These findings, together with the impaired ability to reinfect mTEC cultures compared to naive cells (Fig. 1E and F), suggested the presence of a receptor for influenza virus that was expressed selectively on ciliated airway epithelial cells.

The influenza $\alpha 2,3$ -linked SA receptor is selectively expressed in ciliated cells of mTEC cultures. Influenza virus receptor $\alpha 2,3$ - and $\alpha 2,6$ -linked SA residues can be detected by binding MAA and SNA, respectively (9, 36, 44, 56). Like others (42), we found that both receptors were present on MDCK cells (Fig. 3A). By contrast, mTEC cultures expressed the $\alpha 2,3$ -, but not the $\alpha 2,6$ -linked SA receptor (Fig. 3B). The specificity of $\alpha 2,3$ -linked SA receptor expression was supported by finding a loss of detectable biotinylated MAA binding when mTEC cultures were pretreated with nonconjugated MAA (Fig. 3B, center panel) but not with SNA (data not shown). Consistent with infection and death of ciliated epithelial cells (Fig. 2), the $\alpha 2,3$ -linked SA receptor was selectively expressed on the apical membranes of ciliated epithelial cells based on colocalization of MAA with β -tubulin-IV (Fig. 3C). As expected, infection of ciliated epithelial cells by the H1N1 influenza virus was dependent on the expression of the $\alpha 2,3$ -linked SA receptor (Fig. 3D), demonstrated by abrogated mTEC infection following pretreatment of cultures with MAA (but not SNA) (Fig. 3D; compare center and right panels). Moreover, incubation of mTEC cultures with SeV, a respiratory virus that requires the $\alpha 2,3$ -linked SA receptor (32), resulted in ciliated epithelial cell-specific infection that was blocked by pretreatment with MAA (Fig. 3E). MAA binding was detected in an average of 70.5% of ciliated cells (range, 55

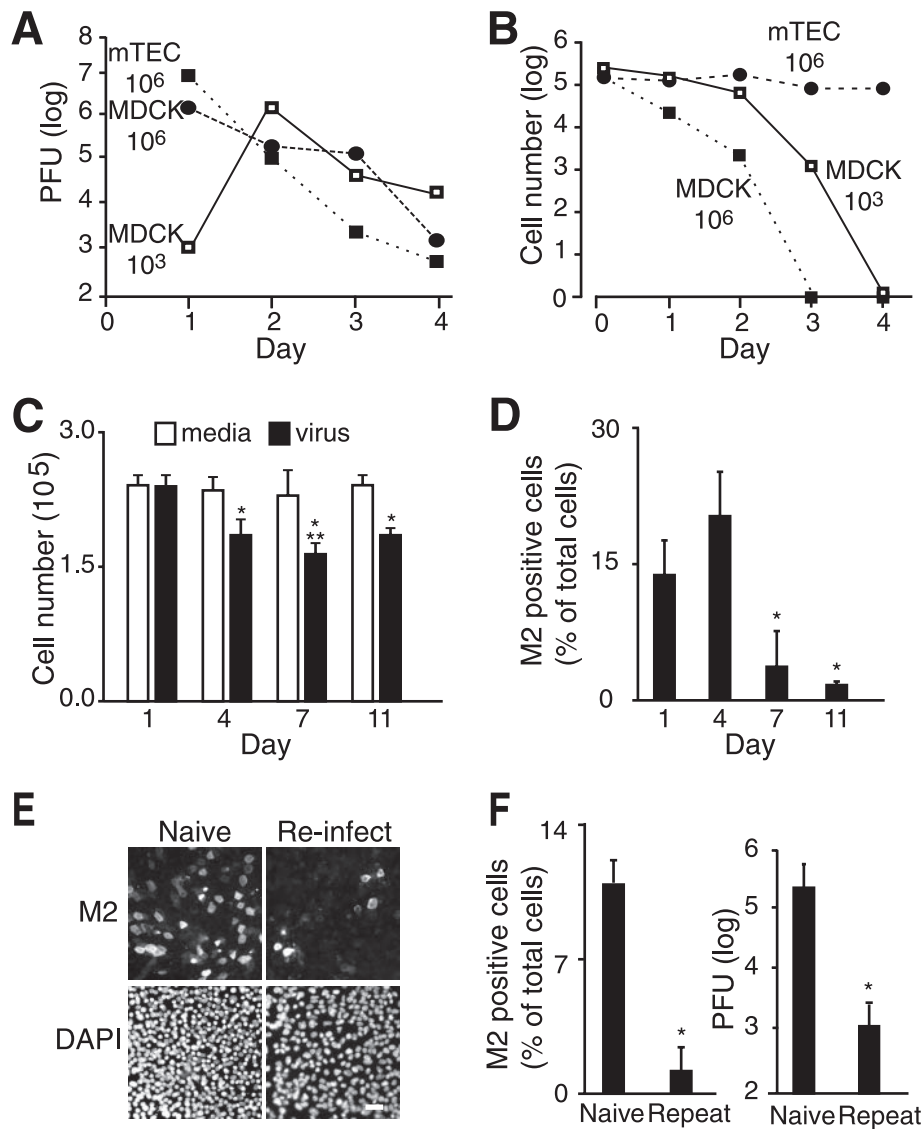


FIG. 1. Influenza virus production and cell survival of MDCK cells and mTEC. (A) Virus production in MDCK cells compared to mTEC cultures. Cells were infected with influenza virus rWSN using the virus titer indicated, and media were collected daily for plaque assay. (B) Cell number following viral inoculation of MDCK cells and mTEC as in panel A. Data are means of duplicate samples from a representative experiment ($n = 3$). (C) mTEC number (means \pm standard deviations) determined by DAPI staining following incubation with medium or influenza virus (1×10^6 PFU/insert, 24 h) as in panel A. There were significant differences ($P < 0.05$) in cell numbers at the indicated days between medium- and virus-treated samples (*) and between virus-treated samples at day 1 and at day 7 (**). (D) Quantification of influenza virus M2 expression in mTEC (means \pm standard deviations). M2 was detected by immunostaining following virus inoculation as for panel C. M2 expression at day 1 and day 4 was significantly different ($P < 0.05$) from that on day 7 and day 11 (*). (E) Reinfection of mTEC cultures. Naive (uninfected) or previously infected (10 days earlier) mTEC were incubated with virus (rWSN, 1×10^6 PFU/sample). One day later, cells were immunostained for M2 expression. (F) M2 expression and virus production (means \pm standard deviations) from cells in panel E were significantly different between samples (*, $P < 0.05$). Data in panels C to F are representative of three independent experiments.

to 79%; three independent preparations), whereas infection resulted in a loss of 85.6% of the ciliated epithelial cells (Fig. 2B). Therefore, the sensitivity of the MAA binding assay for the detection of functional $\alpha 2,3$ -linked receptor was 82% (70.5/85.6%). Collectively, these observations suggested that $\alpha 2,3$ -linked SA was the primary influenza virus receptor in the mouse airway, was present only on the ciliated epithelial cells of the airway, and was an important factor for influenza virus infection.

The $\alpha 2,3$ -linked SA receptor is present on nascent ciliated airway epithelial cells. To further define conditions permissive for mTEC culture infection by influenza virus, we determined the expression of the $\alpha 2,3$ -type receptor during ciliated cell differentiation. Cell differentiation was initiated by the creation of an ALI and was monitored by following the expression of γ -tubulin in basal bodies, an early marker of ciliogenesis (60). At ALI day 0, the cells were undifferentiated and lacked MAA staining, and γ -tubulin was present in only the centrosomes

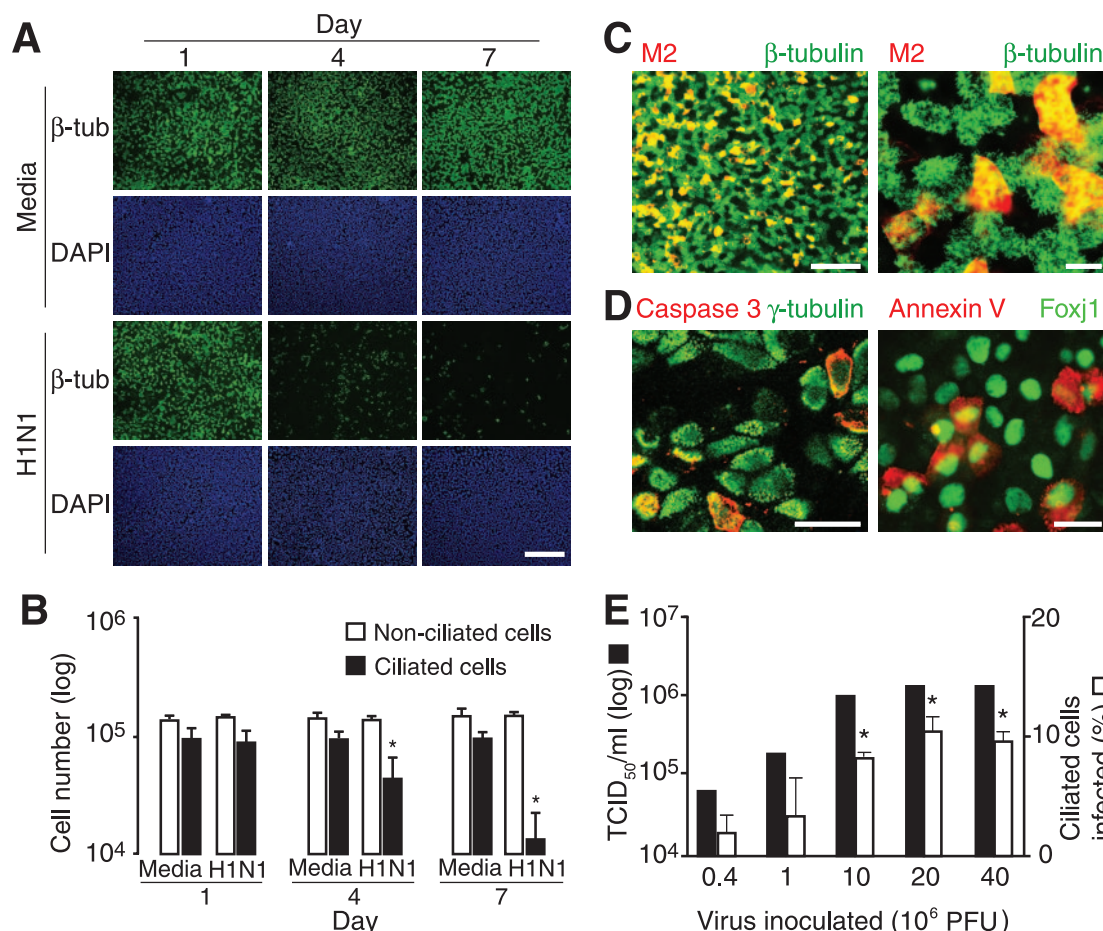


FIG. 2. Influenza virus infects ciliated mouse tracheal epithelial cells. (A) Photomicrograph of representative fields of mTEC cultures inoculated with virus (H1N1, rWSN, 1×10^6 PFU/insert, 24 h), then immunostained with the ciliated cell marker β -tubulin-IV (β -tub), detected with FITC (green), and labeled with DAPI (blue) at the indicated day. Bar, 200 μ m. (B) Quantification of ciliated and nonciliated cells under conditions as in panel A. Cells (identified by DAPI staining) were quantified for the presence or absence of β -tubulin-IV. Shown are means \pm standard deviations from two independent experiments. A significant difference ($P < 0.05$) between ciliated cells incubated with medium versus virus is indicated (*). (C) M2 (red) expression in ciliated cells (β -tub, green) 1 day after infection as in panel A. (Left) Photomicrograph at low magnification (bar, 100 μ m); (right) photomicrograph at high magnification (bar, 10 μ m). (D) Apoptosis in ciliated cells 2 days after infection as in panel A. Shown are photomicrographs of mTEC stained for activated caspase 3 (red) with γ -tubulin (γ -tub, green) and for annexin V (red) with foxj1 (green). Bars, 10 μ m. (E) Effect of virus concentration on mTEC infection. mTEC cultures were inoculated for 1 h with rWSN using the indicated concentration. Twenty-four hours later, cultures were evaluated for virus production by a TCID₅₀ assay, and the percentage of ciliated cells infected (means \pm standard deviations of the percentage of ciliated cells expressing M2) was determined using immunostaining as for panel C. There was no significant difference ($P > 0.05$) in the percentage of infected ciliated cells when virus concentrations equal to or greater than 10×10^6 PFU were used (*).

within the nuclei of all cells. (Fig. 4A). To our surprise, MAA binding was first noted at ALI day 1 (Fig. 4A, center row), prior to the onset of ciliogenesis, which commences at ALI day 2, indicated by the localization of basal bodies at the apical membrane (60). From ALI day 2 until full differentiation of ciliated cells (shown at ALI day 7), colocalization of γ -tubulin and MAA was observed (Fig. 4A, bottom row). Consistent with these observations, the requirement of α 2,3-linked SA for influenza virus infection was confirmed by the inability to infect mTEC at ALI day 0 but permissiveness for mTEC infection at ALI day 1 (Fig. 4B, left panel).

To investigate factors required for the apical presence of the α 2,3-linked SA receptor, we next examined the role of foxj1, a forkhead family member that is required for cilium formation and the apical localization of several proteins (23, 59). In

foxj1-deficient compared to wild-type mTEC cultures, MAA binding and permissiveness for influenza virus infection were similar (Fig. 4C), indicating that foxj1 was not required for α 2,3-type receptor expression and that cilia were not required for infection. To our knowledge, MAA is the earliest known marker of cells that are committed to ciliogenesis.

Ciliated airway epithelial cell-specific expression of the α 2,3-linked SA receptor in vivo. To link our in vitro observations with those in vivo, we found that the α 2,3-linked SA receptor was expressed only in ciliated epithelial cells when evaluated in the airway of mouse lung sections (Fig. 5A). This receptor was most abundant in epithelial cells of the trachea and large airways. Beyond the airway, the α 2,3-linked SA receptor was also found in type II but not in type I alveolar epithelial cells (Fig. 5B). The α 2,6-linked SA receptor was not

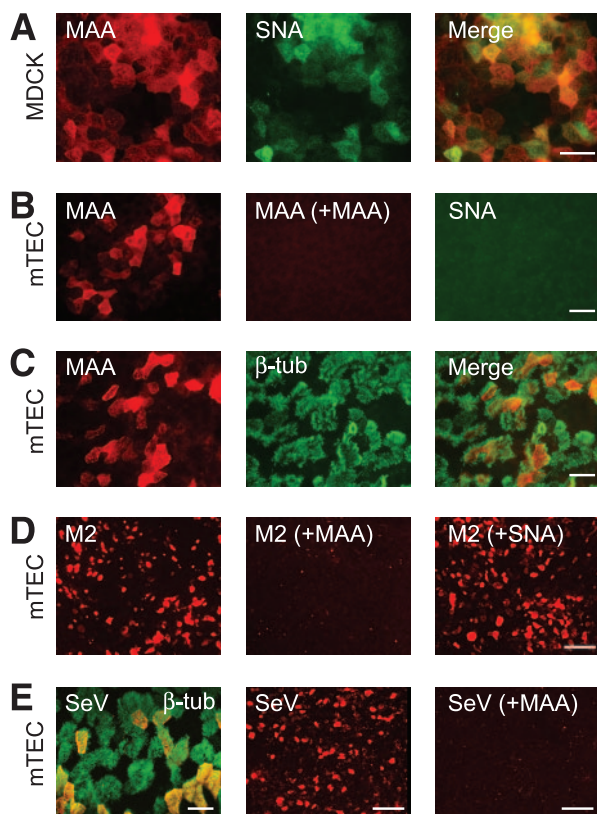


FIG. 3. Selective expression of α 2,3-linked sialic acid in ciliated mTEC. (A) Localization of α 2,3- or α 2,6-linked SA by binding of MAA and SNA lectins, respectively, in MDCK cells. Cells were incubated with biotinylated MAA (red) or SNA (green) and detected by streptavidin-labeled fluorescent dyes. (B) mTEC bind MAA but not SNA. mTEC were either incubated with biotinylated MAA (left) (red), pretreated with unconjugated MAA followed by biotinylated MAA (center) (red), or incubated with SNA only (right) (green). (C) MAA binds ciliated mTEC. mTEC were incubated with biotinylated MAA (red) and immunostained for β -tubulin-IV (β -tub) (green). (D) Effect of lectin pretreatment on influenza virus (rWSN, 1×10^6 PFU/insert) inoculation (low-magnification images). Shown is M2 expression in mTEC after virus inoculation of cells pretreated with medium (left), MAA (middle), or SNA (right). Bar, 100 μ m. (E) Expression of SeV protein in ciliated cells. mTEC were either inoculated with SeV (1×10^5 PFU, 24 h) and then immunostained for SeV protein (red) and β -tub (green) (left) (high magnification) or pretreated with either medium (center) (low magnification [bar, 100 μ m]) or MAA (right) (low magnification [bar, 100 μ m]) and then immunostained for SeV protein. All bars represent 10 μ m except as noted.

detected in the airway or in the alveolar epithelium (data not shown).

In vivo influenza virus infection of the mouse airway is selective for ciliated epithelial cells. To confirm the selectivity of ciliated airway epithelial cell infection in vivo, we titrated influenza virus strain A/WSN/33 in mice (50% lethal dose, 1×10^3 PFU) and identified a sublethal dose that induced an airway bronchiolitis without early death (1×10^2 PFU). In agreement with our in vitro studies, in vivo inoculation resulted in selective infection of ciliated epithelial cells in the airway (Fig. 5C). At day 9 after infection, there was a decrease in ciliated epithelial cells and M2 expression in affected airways that was consistent with virus clearance, weight recovery, and

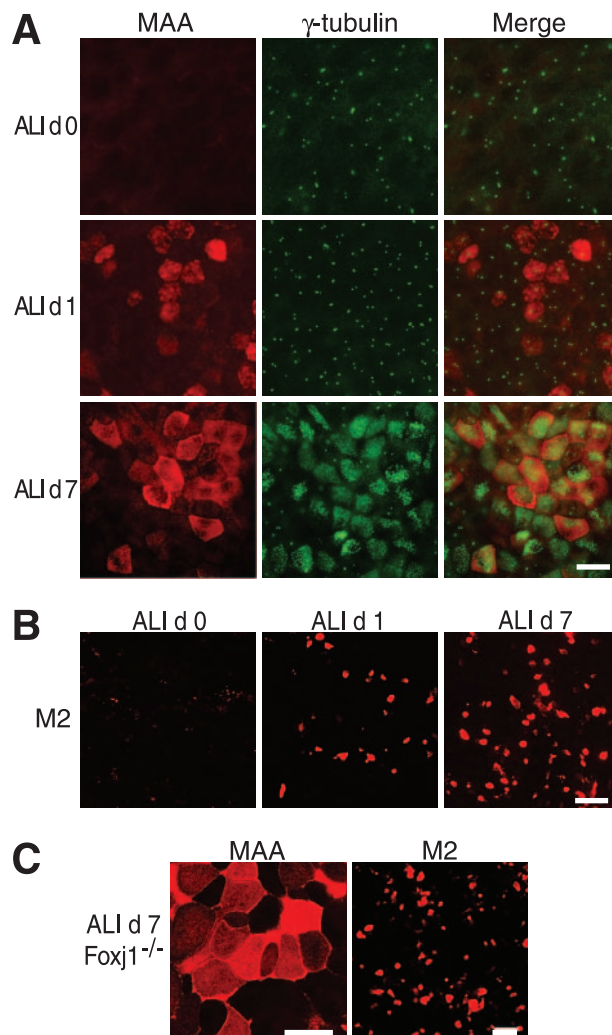


FIG. 4. Expression of α 2,3-linked sialic acid in preciliated mTEC. (A) MAA binding during differentiation of mTEC cultures. mTEC were cultured until confluent, then differentiated using ALI conditions. At the indicated ALI day (d), mTEC were incubated with biotinylated MAA (red) and immunostained with γ -tubulin (green), a marker of ciliated cell basal bodies. MAA binding was detected at ALI day 1, prior to the presence of organized apical basal bodies shown at ALI day 7. Bar, 10 μ m. (B) Effect of mTEC differentiation on influenza virus infection. Shown is M2 expression from the indicated ALI day in mTEC infected with rWSN (1×10^6 PFU/insert, 24 h). Bar, 100 μ m. (C) Virus infection of mTEC cultures from cilium-deficient *Foxj1*^{-/-} mice. (Left) MAA binding was detected as for panel A. Bar, 10 μ m. (Right) *Foxj1*^{-/-} mTEC were infected with virus and M2 detected as for panel B. Bar, 100 μ m.

epithelial cell repair (29, 30). (Fig. 5C and D). Although the airway was the primary site for infection, some regions of the lung showed infection of type II alveolar epithelial cells, consistent with localization of the α 2,3-type receptor in these cells (Fig. 5D inset). M2 expression was also identified in alveolar macrophages and sloughed airway epithelium (Fig. 5D).

Cell-selective expression of influenza virus receptors in human airway epithelial cells. Although both the α 2,3- and α 2,6-type receptors have been found in the human lung, descriptions of cell-specific localization vary (2, 36, 45). These conflicting studies led us to use both ALI-differentiated hTEC

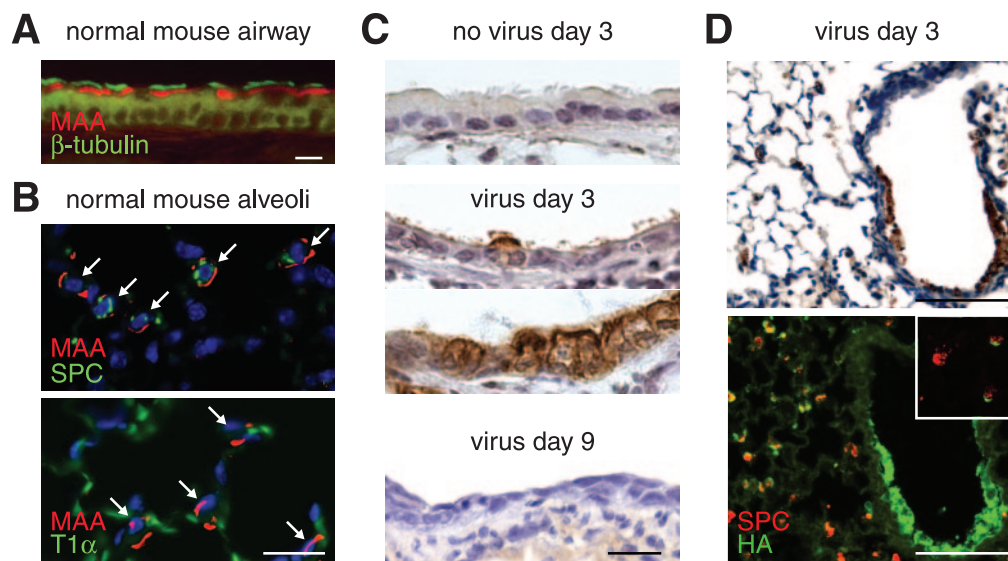


FIG. 5. Selective expression of the α 2,3-linked SA receptor in mouse lungs and influenza virus infection in vivo. (A) Expression of the α 2,3-linked SA receptor (red) in ciliated airway epithelial cells (green) in fixed, paraffin-embedded sections (5 μ m) from the mouse large airway. (B) The α 2,3-linked SA receptor (red) is expressed in type II (SPC, pro-surfactant protein C, green) (top) but not in type I (T1 α , green) (bottom) alveolar epithelial cells. Arrows indicate cells with MAA staining. (C) M2 expression in ciliated cells from lungs of mice inoculated with influenza virus A/WS/33 intranasally using 10^4 PFU analyzed at the indicated day for M2 expression by the immunoperoxidase method (brown) and counterstained with hematoxylin. Two different examples of infected ciliated cells at day 3 are shown. (D) Low-magnification image of virus infection (as in panel C) in large airways and type II alveolar epithelial cells detected by immunoperoxidase (M2) (top) and immunofluorescence (HA, green; SPC, red) (bottom). (Inset) SPC expression in type II cells. Bars for panels A to C, 10 μ m; bar for panel D, 100 μ m.

cultures and human lung sections to compare our mouse data to data for humans. In hTEC cultures, the α 2,3-linked SA receptor was found only in ciliated cells (mean, 72.9%; range, 66 to 84.6%) and localized to the apical membrane (Fig. 6A, left panel). However, α 2,6-linked SA was expressed in both ciliated (mean, 54.5%; range, 37.0 to 81.0%) and nonciliated (mean, 35.3%; range, 19.0 to 53.0%) cells (Fig. 6A, center panel). Approximately one-third of ciliated cells expressed both types of receptors (Fig. 6A, right). A similar pattern of localization for both types of receptors was found in lung sections ($n = 5$ individuals) (Fig. 6B and C). Previously not noted by others, the α 2,3-type receptor was also expressed in a subpopulation of basal cells (Fig. 6B). Interestingly, the non-ciliated cells that expressed the α 2,6-type receptor had the typical appearance of goblet cells (Fig. 6C). Indeed, colocalization of SNA with nonciliated cell-specific markers for Clara (CCSP) or goblet (MUC5AC) cells revealed the presence of an α 2,6-type receptor only in goblet cells (Fig. 6D). Both receptors were most abundant in the bronchi (large airways) but were found in epithelial cells in smaller airways. As in the mouse lung, the α 2,3-type receptor (but not the α 2,6 type) was found in human alveolar type II epithelial cells (Fig. 6E). Collectively, these findings suggested that extension of the cell tropism of human influenza viruses via the α 2,6-linked SA could result in significant impairment of the mucociliary apparatus by targeting both ciliated and goblet cells.

Airway epithelial cell-specific tropism of influenza viruses in hTEC. To functionally identify the cell-specific localization of receptors in human airway cells, we used a mouse-adapted (rWSN) and a human strain (low passage; A/California/7/2004) to compare infection in mTEC and hTEC cultures. As in mTEC cultures (Fig. 2), inoculation of hTEC with rWSN re-

sulted in infection of ciliated epithelial cells only (cell types that express only the α 2,3-type receptor), even if the MOI was increased fourfold, based on colocalization of M2 and β -tubulin-IV (Fig. 7A). Likewise, inoculation of hTEC cultures by A/California/7/2004 resulted in infection of ciliated and non-ciliated cells (cell types that express the α 2,6-type receptor) (Fig. 7B, left, and 7C). However, this strain did not infect mTEC, even if the MOI was increased up to eightfold (Fig. 7C, left). In agreement with the failure to infect mTEC cultures with A/California/7/2004, in vivo inoculation of mice (10^3 to 10^5 PFU; $n = 30$) resulted in no evidence of disease (no weight loss) and no death. Although we recovered virus from lung homogenates at 30 min, we failed to detect virus at days 2, 3, and 6 postinoculation using either the TCID₅₀ assay or virus immunolabeling of lung epithelium (data not shown).

To determine the capacity of other human strains to infect both mice and humans, we evaluated the H3N2 human strain rUdorn. Inoculation with this virus resulted in productive infection of mTEC ciliated cells, suggesting infection of α 2,3-type receptor-expressing cells (Fig. 7C). In hTEC cultures, this strain infected both ciliated and nonciliated cells (Fig. 7C), suggesting the additional ability to infect via the α 2,6-type receptor. Thus, some human strains retain the capacity to infect cells via the α 2,3-type receptor as well as the α 2,6-type receptor present in the nonciliated cells.

The majority of nonciliated epithelial cells in the human airway are basal cells, Clara (secretory) cells, or goblet cells (20). Interestingly, the goblet cells, which have been hypothesized to produce mucous as an antiviral defense, were themselves infected by A/California/2002 (Fig. 7B, right). Infection of goblet cells was more frequent in A/California/2002 virus-inoculated hTEC cultures that were treated with interleukin-13

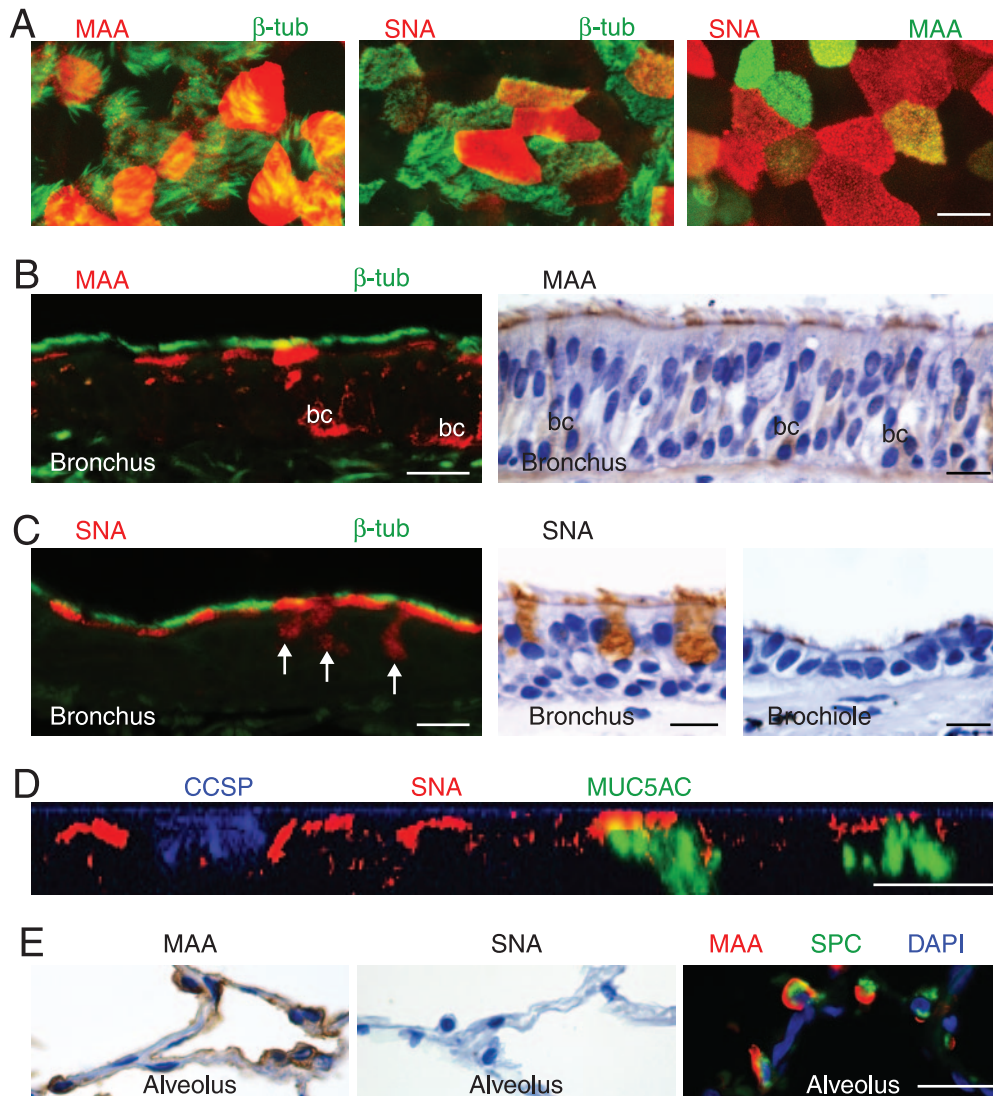


FIG. 6. Cell-selective localization of influenza virus receptors in human airway epithelial cells. (A) hTEC cultures were either incubated with biotinylated MAA (red) (left) or SNA (red) (center) and colocalized with the ciliated cell marker β -tubulin (β -tub, green) or incubated with both MAA (red) and SNA (green) (right). (B) Photomicrographs of MAA binding in sections of human lung detected by immunofluorescence (left) and immunoperoxidase (right). Expression of MAA in ciliated cells and a subpopulation of basal epithelial cells (bc) is shown. (C) Photomicrographs of SNA binding in ciliated and goblet cells (arrows, left) using methods as in panel B. (D) Representative reconstructed (x,z axis) confocal microscopy image of hTEC cultures showing localization of SNA binding (red) with goblet cells (MUC5AC, green) but not with Clara cells (CCSP, blue). (E) Detection of receptors in alveoli of human lung sections with MAA (left) and SNA (center) using immunoperoxidase and colocalization of MAA (red) with the type II alveolar epithelial cell marker SPC (green) using immunofluorescence (DAPI, blue) (right). Panels A to E are representative of expression in at least five different donors. Bars, 10 μ m.

to induce goblet cell metaplasia and decrease the number of ciliated cells (27) (data not shown). This confirmed that goblet cells (a cell type not normally present in the mouse lung) could support productive infection. Thus, differences in mouse and human strain tropism and cell receptor specificity are important factors for consideration of models used to investigate influenza pathogenesis.

DISCUSSION

Successful infection of the respiratory tract by viruses is the result of a balance between pathogen virulence, host receptor expression, innate responses, and acquired responses. Mouse

models have played an important role in uncovering many of these mechanisms, and the ability to manipulate the mouse genome provides a powerful tool for future studies. To extend findings from mice to human disease, it is critical to characterize factors important for infection, including virus-specific receptors, in each host. We characterized influenza virus receptors *in vitro* using differentiated mouse tracheal epithelial cells, lung sections, and an *in vivo* mouse model, and we determined the human relevance using a human *in vitro* airway epithelial cell model and human lung sections. We found these species had shared patterns of expression of only the α 2,3-linked SA receptor, each restricted to ciliated airway epithelia,

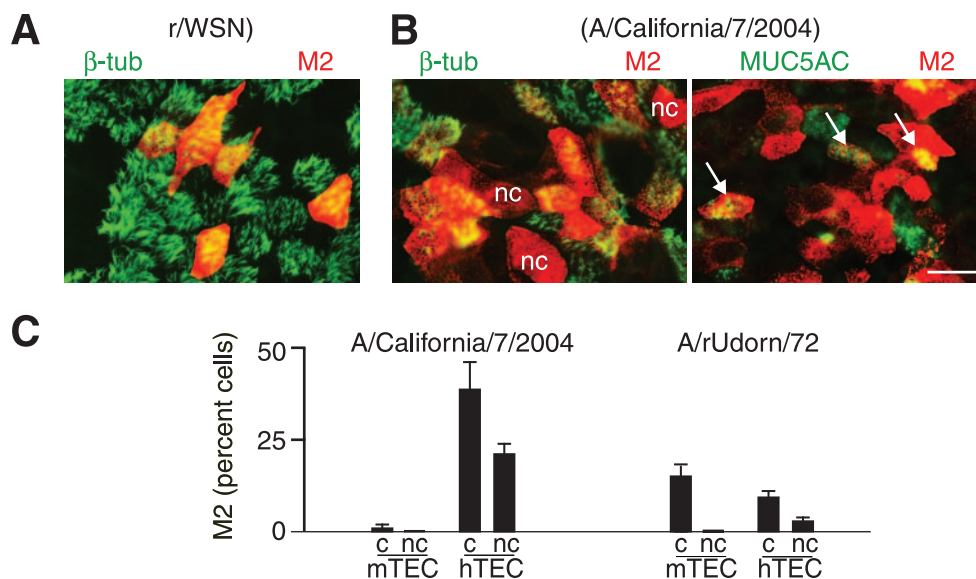


FIG. 7. Human airway epithelial cell-specific tropism of influenza virus strains that preferentially bind to α 2,3- or α 2,6-linked SA. hTEC cultures were incubated with the indicated virus strain and analyzed by immunostaining. (A) rWSN (H1N1; 1×10^6 PFU/insert; 22 h) virus (M2, red) infects ciliated cells (β -tub, green) only. (B) A/California/7/2004 (H3N2; 2×10^6 PFU/insert, 22 h) virus infects ciliated cells and nonciliated (nc) cells identified as goblet cells (arrows). Cells expressing M2 (red) were colocalized with β -tubulin (green) (left) or MUC5AC (green) (right). (C) Quantification of M2 expression in ciliated (c) and nonciliated (nc) mTEC or hTEC infected with A/California/7/2004 as in panel B and A/rUdorn/72 (2×10^4 PFU/insert, 20 h). Percentages of infected cells are means \pm standard deviations.

type II alveolar epithelial cells, and some basal cells. In contrast, our studies showed that the α 2,6-linked SA receptor was not expressed in mice but was present in human ciliated airway epithelial cells and also goblet (mucous) cells, a population not present in the mouse airway (20). Differences in host receptor distribution may be important factors in consideration of virus pathogenesis among different strains when evaluated in animal models.

Prior studies have not defined types and cell-specific localization of influenza virus receptors in epithelial cell populations of the mouse lung. One study suggested that the mouse lung was preferentially infected by virus strains that bind the α 2,6-type receptor (13), while another showed that the α 2,6-type receptor was absent, based on SNA binding (11). Our finding that α 2,3-linked SA is the only influenza virus receptor in the mouse lung was supported by the use of epithelial cell-specific markers, lectin binding, and functional studies in relevant in vitro and in vivo mouse models. These functional studies included the use of different virus strains that variably infected mouse and/or human epithelial cells, also suggesting differences in receptor populations. Together, these studies indicated that α 2,3-type receptor localization was in ciliated airway and type II alveolar epithelial cells. The functional studies showed that some human strains infected mTEC (e.g., A/rUdorn/72 and A/HK/1/68), suggesting the retention of the capacity to utilize the α 2,3-type receptor. It is possible that mouse-adapted strains also retained α 2,6-type binding capacity; however, further studies are required to elucidate this feature.

Descriptions of the localization of influenza virus receptors in humans have differed. An early report described selective MAA binding in goblet cells and SNA binding in ciliated cells but considered weak SNA binding in goblet cells to be an

artifact (2). While others found that SNA binds only nonciliated cells, thought to be Clara cells (36), we detected SNA in both ciliated cells and nonciliated cells that we identified as goblet cells. The localization of SNA in both ciliated and nonciliated cells has also been noted in some studies (11, 61). Our finding that MAA was selective for ciliated cells is in agreement with recent studies of hTEC cultured using ALI conditions (36, 61). Identification of MAA binding within the alveolar compartment is also consistent with a recent report (45). These findings indicate that receptor expression is cell specific, but differences may reflect limitations of lectin-based detection.

Methods for detection of specific receptors are limited to the use of lectins and association with variations of patterns of infection in mouse compared to human cells. Lectins are widely used as a method for detection of influenza virus receptors, and we found that MAA was sensitive and specific for identification of the receptor in ciliated epithelial cells. However, we found that lectins from different manufacturers differ in the capacity to bind different cell types, particularly the alveolar epithelia. Variation among reports of receptor localization may also be due to differences in conditions used for lectin binding, preservation of glycosylated forms, and variations in sialyltransferase gene expression among tissue samples used. Alternatively, virus binding to fixed human tissues has been used, particularly where cell-specific localization in natural infection is difficult to evaluate (52). Consistent with our findings, these studies have shown that an H3N2 virus binds to the ciliated cells of the human trachea (52). Complex variations in carbohydrate structures may also contribute to binding of viruses and account for differences in infection of human and avian strains; however, this work is still evolving (14).

The regulation of cell-specific expression of receptors in

mice and humans is not well understood. We found that expression of the α 2,3-linked SA receptor was regulated during differentiation. This receptor was present in preciliated mouse cells (prior to cilium growth) and did not require foxj1, a factor required for localization of other apical membrane proteins (23). Regulation of cell-specific expression of receptors also appears complex, since we and others (61) found MAA and SNA did not bind all ciliated cells, and approximately 30% of ciliated hTEC bound both MAA and SNA (Fig. 6A). The mouse and human sialyltransferases capable of α 2,3- and α 2,6-SA linkages are part of a large family (18, 33). Although α 2,6-linked SA was not found in mouse lung, sialyltransferases capable of performing this function were found to be expressed in our microarray studies of mTEC cultures (A. Ibricevic and S. L. Brody, unpublished observations). Thus, it is tempting to hypothesize that SNA localization might be related to binding of specific mucin proteins, since some of these glycosylated proteins are not present in the mouse but are expressed in a cell-specific fashion in the human airway. Attractive substrates for sialylation are the apically displayed tethered mucins (26). It is also possible that secreted mucins, such as the goblet cell marker MUC5AC, could also bind α 2,6-SA. But we found that interleukin-13 induction of MUC5AC in mouse airway epithelial cells did not result in SNA binding in goblet cells (Ibricevic and Brody, unpublished). Additionally, it may be that species-specific regulation of sialyltransferase has developed evolutionarily in specific cell types (11). Among primates studied, it was reported that only great apes express α 2,6-linked SA, and then only in goblet cells (11). Interestingly, pigs and chickens (but not ducks) have both receptor types, a feature that may play important roles in reassortment and transmission of influenza virus (12, 24). How sialylation patterns and SA expression evolved relative to a role for these animals as reservoirs of human viruses is not known.

Our observed differences in cell-specific localization of influenza virus receptors have several important implications for the study of influenza virus strains in mice and humans. First, expression of only the α 2,3-type receptor in the mouse provides one explanation for the failure of human strains with a preference for the α 2,6-type receptor, but very low affinity for the α 2,3 type, to infect mice. This was consistent with our observations that the human strain A/California/7/2005 was less virulent in mTEC cultures (even when a higher titer was used) than rUdorn or A/HK/1/68 strains. Second, differences in mouse and human airway epithelial cell populations must be considered if the mouse is infected with strains that can bind both of the receptor types. In mice and humans, 40 to 50% of the cells in contact with the airway lumen are ciliated and thus can be infected. However, mice do not have goblet cells, which account for 10 to 20% of epithelial cells in the human airway and are permissive only to human strains (6, 20). Thus, injury, cell-specific innate epithelial responses, and subsequent viral kinetics may differ between species. Third, the localization of the α 2,3-linked SA receptor in some basal cells of the airway may have important implications for airway repair, since this cell type has been described as pluripotent or stem cell-like for regeneration of new epithelial cells in mice or humans with inflammatory airway disease (22, 54). This may be particularly important in the human, where the basal cell is more abundant than in the mouse (20). Fourth, there is a pressing need for

understanding how viruses mutate to favor infection of different species. The lack of the α 2,6-linked SA receptor in the mouse also indicates that this species is an ideal model for evaluation of the adaptation process. Fifth, knowledge of the shared expression of α 2,3-linked SA in human and mouse ciliated epithelial cells could improve understanding of other pathogens that bind this motif (1). Finally, targeting a specific cell type for gene or protein therapy could be augmented in mice by use of a ligand for the α 2,3-linked SA receptor (38).

The emergence of avian influenza viruses that bind only to the α 2,3-linked SA receptor and the sole expression of this receptor in the mouse lung raise the possibility that the mouse could be used to study these strains. Nonadapted H5N1 avian strains that bind only α 2,3-linked SA can infect mice (31); however, the pathogenicity of H5N1 in mice varies widely (10). In the mouse, H5N1 virus attachment has been shown in the ciliated cells of the trachea and type II pneumocytes (52). In human cells, virus production following in vitro infection of primary culture airway and alveolar epithelial cells was robust (8), consistent with our localization of the α 2,3-type receptor. However, one human autopsy study (51) and ex vivo infection of human tissues (45) suggest that H5N1 preferentially infects alveolar compared to airway cells. This may be difficult to determine in the mouse model, where experimental virus delivery to the alveolus following intranasal inoculation may be low due to smaller dimensions that limit airflow compared to that for humans. Issues concerning cell-specific infection and putative influenza virus receptors, together with the high mortality associated with H5N1 viruses, indicate an urgent need for assessment of factors that contribute to infection in humans. In vitro cultures of primary airway cells or in vivo mouse models may be useful for study of these viruses and for development of effective animal and human vaccines and therapies against new strains of influenza virus.

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